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Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments

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ABSTRACT

The largely unused uracil-excision molecular cloning technique has excellent features in most aspects compared to other modern cloning techniques. Its application has, however, been hampered by incompatibility with proof-reading DNA polymerases. We have advanced the technique by identifying PfuCx as a compatible proof-reading DNA polymerase and by developing an improved vector design strategy. The original features of the technique, namely simplicity, speed, high efficiency and low cost are thus combined with high fidelity as well as a transparent, simple and flexible vector design. A comprehensive set of vectors has been constructed covering a wide range of different applications and their functionality has been confirmed.

INTRODUCTION

The principle of uracil-excision based cloning, which was conceived in the early 1990s (1,2), has in 2003 resulted in a commercial method, the USER™ (uracil-specific excision reagent) cloning technique (New England Biolabs). Briefly, in the commercial technique the cloning event relies on the ability of 8 nt long complementary 3′ overhangs generated at the ends of, respectively, a PCR amplified DNA fragment and a linearized destination vector to make a stable hybridization product, which can be used to transform host organisms without prior ligation. These overhangs are generated on PCR fragments by placing a single uracil residue in each primer used to amplify the target DNA and subsequently treating the resulting PCR product briefly with uracil DNA glycosylase (2) and DNA glycosylase-lyase Endo VIII. These enzymes, which are included in the USER™ enzyme mix, remove the two single uracil residues and enable the dissociation of the single-stranded fragments lying upstream from the cleavage sites (exemplified in Figure 1).

The commercial USER™ technique enjoys a large number of advantageous features. Most prominent is its simplicity. Primers for amplifying PCR fragments need only to have 8 bp tails added to their specific sequence and the vector design involves simple insertion of a small cassette into the multiple cloning site of already established vectors. Another strong feature of the technique is the strength by which the long overhangs on PCR fragment anneal to the complementary overhangs on the vector to generate recombinant DNA molecules in a ligation independent manner at a very high efficiency. Furthermore, the technique involves minimal handling and is very robust as PCR products at a wide range of concentrations can be mixed directly with USER™ enzyme mix and a predigested stock of linearized vector without purification or further modifications to give the recombinant molecules. This makes the technique highly suitable for single as well as high-throughput cloning experiments of PCR fragments.

In spite of these advantages, we have not found a single published work using the commercial USER™ technique, which indicates that the method is largely unused. This is most likely due to an incompatibility between the technique and proof-reading DNA polymerases, which stall at uracils present in DNA templates (3,4). Consequently, only the low-fidelity *Taq* based polymerases have been applicable, which has made the cloning of error-free DNA difficult. This negates all advantageous features and practically renders the technique close to useless.

In this study, we have identified a proof-reading DNA polymerase that is compatible with the uracil-excision based cloning technique and we provide an improved, versatile vector design strategy. The advances allow the great potential of the technique to be fully exploited.

MATERIALS AND METHODS

PCR conditions

PCR with the following DNA polymerases: HotMaster™ *Taq* DNA Polymerase (Eppendorf), Platinum® *Taq* DNA

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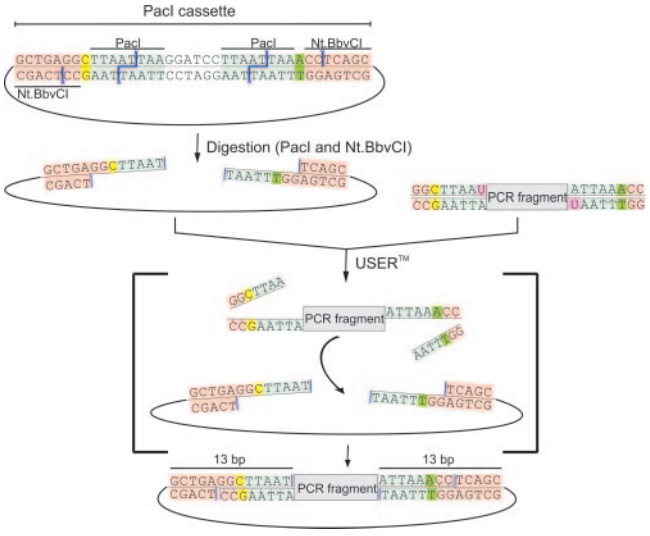

Figure 1. Overview of the USER cloning technique. A PacI cassette containing USER vector (upper left corner) is digested with PacI and Nt.BbvCI to generate 8 nt single-stranded 3′ overhangs. A PCR fragment amplified with compatible uracil-containing primers by the PfuTurbo® C_x Hotstart DNA polymerase is mixed with USER™ enzyme mix (removing uracils, pink) and the linearized vector. The mixture is incubated 20 min at 37°C and 20 min at 25°C, and the hybridized product is ready to be transformed into *E.coli* without prior ligation. Nt.BbvCI recognition sites are marked in tan, PacI recognition sites are marked in light blue. Yellow and green mark single base differences between the generated 3′ overhangs, which are responsible for the directional insertion of the PCR fragment.

Polymerase (Invitrogen), Pwo DNA Polymerase (Roche), Phusion™ DNA Polymerase (Finnzymes), and PfuTurbo® C_x Hotstart DNA polymerase (PfuCx) (Stratagene) was performed according to manufacturers' instructions on pBAD-TOPO® (Invitrogen) containing the gene, At5g43440 (Accession no. AY143873). The following primer combinations used were: Uracil-free forward primer: (5′-ATGACAGAAAAATCTGCAGAACT-3′), uracil-free reverse primer: (5′-TCATATCCGGAACTTAGACAA-3′), uracil-containing forward primer: (5′-GGCTTAAUATGACAGAAAAATCTGCAGAACT-3′) and uracil-containing reverse primer: (5′-GGTTTAAUTCATATCCGGAACTTAGACAA-3′). All oligonucleotides used in this study were acquired from Invitrogen.

Generation of USER compatible vectors

Chemically synthesized oligonucleotide cassettes composing the PacI cassette were cloned into various destination vectors using standard molecular biology methods. Forward strand: (5′-restriction enzyme site + GGCTGAGGCTTAATTAAGGATCCTTAATTAAACCTCAGC-3′) and reverse strand: (5′-restriction enzyme site + GGCTGAGGTTTAATTAAGGATCCTTAATTAAGCCTCAGC-3′). The resulting USER compatible constructs were verified by sequencing (for extended tables including the corresponding oligonucleotides used to construct each USER vector described in this study see Supplementary Data).

Preparing USER vectors for cloning

The PacI cassette-containing vector is prepared for cloning by digesting with PacI and subsequent nicking by Nt.BbvCI. Prolonged digestion is performed to ensure complete digestion, which minimizes false positive colonies and increases cloning efficiency. A total of 5 μg plasmid DNA of the constructed vectors were digested with 40 U PacI (New England Biolabs) overnight at 37°C in a total volume of 200 μl. Additional 20 U of PacI were added the next day together with 20 U Nt.BbvCI (New England Biolabs), and the digestion was incubated for 2 h at 37°C. The linearized vector was purified using the Qiagen PCR purification kit.

Insertion of PCR fragments into USER vectors

Any DNA fragment to be inserted into any of our PacI cassette-containing USER vectors was PCR amplified with primers, which in addition to the sequence specific to the target DNA fragment contained a tail of 8 nt (see below). Forward primer: (5′-GG**C**TTAAU + sequence complementary to target DNA-3′), Reverse primer: (5′-GG**T**TTAAU + sequence complementary to target DNA-3′). For amplifying genes to be inserted into C-terminal fusion vectors (see below) a reverse primer was used, which replaced the stop codon with two cytidine residues. C-terminal fusion reverse primer: (5′-GGTTTAAU + CC + sequence complementary to target gene upstream of stop codon-3′). PCR was performed with PfuCx DNA polymerase according to manufacturer's instructions. Subsequently, a mixture of PCR product, 1 U USER™ enzyme mix (New England Biolabs), and PacI/Nt.BbvCI digested USER vector was incubated 20 min at 37°C followed by 20 min at 25°C and finally transformed into chemically competent *Escherichia coli* cells (do not use electroshock transformation). The reaction is very robust and has high efficiency when as little as 0.01 pmol of linearized vector and >0.02 pmol of PCR fragment are used. In practice, the concentrations of linearized vector and PCR product were estimated by gel electrophoresis and mixed in a 1:10 molar ratio. This ratio is the optimal ratio between vector and PCR fragment, which gives the largest number of recombinant colonies with the lowest proportion of false positives (http://www.neb.com/nebecomm/ManualFiles/manualE5500.pdf).

In the text, the term 'USER cloning' will be used to describe the steps that involve (i) PCR amplification of target DNA fragment with PfuCx using the primers described above (ii) mixing of PCR fragment with linearized vector and USER™ enzyme mix and (iii) transformation of *E.coli*.

Generation of USER compatible translational fusion vectors by sequential USER cloning of multiple inserts

PacI cassettes were regenerated when USER cloning into the PacI cassette of existing USER vectors by including 25 bp of the 38 bp PacI cassette sequence in the end of inserted DNA fragments. The 25 bp regenerate the PacI cassette either upstream or downstream from the inserted DNA fragment depending on, in which end of the DNA fragment the 25 bp is incorporated.

Vectors for N-translational fusions were made by USER cloning tags into USER compatible vectors while regenerating the PacI cassette downstream of the tag. PCR amplification of the tags was performed with PfuCx DNA polymerase as described above using a normal forward primer (5′-GGCTTAAU + tag ORF specific sequence-3′) and a reverse primer which included 25 additional bases (underlined) that

regenerate the PacI cassette downstream of the tag upon insertion (5′-GGTTTAAUTAAGGATCCTTAATTAAGCCTCAGC*CC* + tag ORF specific sequence-3′). Between the 25 bases and the tag ORF specific sequence, two cytidine residues have been added (italic) to bring the subsequently inserted gene into the same reading frame as the tag. Short tags can be synthesized as oligonucleotides and USER cloned into PacI cassette containing vectors to create N-terminal translational fusions. Forward strand: (5′-GGCTTAAU + tag ORF coding strand + *GG*GCTGAGGCTTAATTAAGGATCCTTAATTAAACC-3′) and reverse strand: (5′-GGTTTAAUTAAGGATCCTTAATTAAGCCTCAGC*CC* + tag ORF complementary strand + ATTAAGCC-3′).

For USER cloning genes into N-terminal fusion vectors, genes were PCR amplified with normal forward primer: (5′-GG**C**TTAAU + sequence complementary to target DNA-3′) and normal reverse primer: (5′-GG**t**TTAAU + sequence complementary to target DNA-3′) and USER cloned into the vectors as described above.

Vectors for C-terminal translational fusions were made by inserting tags that were PCR amplified using a forward primer: (5′-GGCTTAAUTAAGGATCCTTAATTAAACCTCAGC + tag ORF specific sequence-3′), which regenerate the PacI cassette upstream of the tag and a normal reverse primer: (5′-GGTTTAAU + tag ORF specific sequence) or by inserting chemically synthesized oligonucleotides, forward strand: (5′-GGCTTAAUTAAGGATCCTTAATTAAACCTCAGC + tag ORF + ATTAAACC-3′) and reverse strand: (5′-GGTTTAAU + tag ORF complementary strand + GCTGAGGTTTAATTAAGGATCCTTAATTAAGCC-3′). For USER cloning genes into C-terminal fusion vectors, the native stop codon of the gene was replaced with two cytidine residues (in the antisense strand) to bring the inserted gene into reading frame with the downstream tag. Thus, genes were PCR amplified with a normal forward primer: (5′-GG**C**TTAAU + sequence complementary to target DNA-3′) and reverse primer: (5′-GG**T**TTAAU + CC + sequence complementary to target gene upstream of stop codon-3′).

USER cloning and expression of cyano fluorescent protein (CFP) *in planta*

CFP was USER cloned into the plant specific pCAMBIA-230035Su vector (Table 1) as described above with the primers: UCFP-F: (5′-GGCTTAAUATGGTGAGCAAGGGCGAGGAG-3′) and UCFP-R: (5′-GGTTTAAUTTACTTGTACAGCTCGTCCATG-3′).

The resulting vector construct was subsequently transformed by electroporation (5) into *Agrobacterium tumefaciens* strain C58 (6). *Arabidopsis thaliana* plants were transformed by the floral dip method (7).

USER cloning and expression of AtSTP1 in *Xenopus* oocytes

AtSTP1 was USER cloned into the *Xenopus laevis* expression vector, pNB1u (Table 1), as described above. PCR amplification from pda05545 (Riken BRC) was performed with the primers: USTP1-F: (5′-GGCTTAAUATGCCTGCCGGTGGATTC-3′) and USTP1-R: (5′-GGTTTAAUTCAAACATGCTTCGTTCC-3′). The resulting pNB1u-STP1 plasmid DNA was linearized with NotI and subsequently *in vitro* transcribed using the T7 mMessage mMachine kit (Ambion®) according to the manufacturer's instructions. Oocytes were prepared as described previously (8), and subjected to injection of 50 ng cRNA. Oocytes were incubated for 2–3 days at 17–18°C, and assayed for transport uptake activity as described previously (9).

RESULTS

Identification of a uracil compatible proof-reading DNA polymerase

In a literature search, we sought to understand the structural basis for the uracil inhibition of proof-reading DNA polymerases and the resulting incompatibility with uracil-containing primers. We discovered that a non proof-reading version of the *Pyrococcus furiosus* (*Pfu*) polymerase recently was engineered to read through uracils present in DNA templates (10). A version with proof-reading activity of this polymerase was later found to be commercially available as the PfuTurbo® C_x Hotstart DNA polymerase. We tested the ability of the non proof-reading DNA polymerases Hotmaster *Taq*, and *Taq*, as well as the proof-reading DNA polymerases; Phusion, Pwo and PfuCx to perform with uracil-free and uracil-containing primers (Figure 2a). The experiment showed that whereas all five polymerases accept uracil-free primer combinations, only the non-proof-reading *Taq* and the proof-reading PfuCx polymerases were able to exponentially amplify templates using uracil-containing primers.

Vector design of USER compatible vectors

Vector design for the commercial USER™ cloning technique involves insertion of a 42 bp cassette which contains two XbaI restriction recognition sites flanked by two Nt.BbvCI nicking recognition sites into the multiple cloning sites (http://www.neb.com/nebecomm/ManualFiles/manualE5500.pdf). Subsequent digestion of the constructed vector with these two enzymes generates a linearized fragment with 8 nt 3′ overhangs at each end (Figure 1). To be applicable with the uracil-based cloning technique, it is required for the restriction enzyme used in the cassette to cut after a thymidine residue in their recognition sequence. This thymidine residue will be the last residue in the 8 nt 3′ overhangs in the vector and will correspond to the uracil in the PCR primers (Figure 1). We found that PacI, PmeI, AsiSI and SwaI fulfill this requirement and, in addition, these all have 8 bp recognition sequences which statistically occur less frequently than the 6 bp restriction site of XbaI. We designed a 38 bp cassette that has two PacI sites flanked by two Nt.BbvCI sites at its core (PacI cassette, Figure 1, left panel). The sequence of the 8 nt 3′ overhangs generated in USER vectors after digestion is mostly dictated by the recognition sites of the nicking and restriction enzymes used in the cassette. Since the same nicking and restriction recognition sites are used in both halves of the cassette, directional insertion of DNA fragments is ensured by varying the nucleotides lying between the respective recognition sites. In the XbaI-containing cassette, three variable nucleotides lie between the recognition sites of Nt.BbvCI and XbaI. In our PacI cassette, only one variable nucleotide lies between the Nt.BbvCI and PacI recognition sites (Figure 1). This could potentially

Table 1. List of generated USER vectors

#	USER vector	Origin of vector	Organism	Promoter	Description of application
1	pPICZAu (3.6 kb)	pPICZA (Invitrogen)	*P.pastoris*	AOX1	Constitutive expression. Zeocin resistance.
2	pNB1u (3 kb)	pNB1 (15)	*X.laevis* oocytes	T7	Constitutive expression. Xenopus β-globin UTRs. Ampicilin resistance
3	pCAMBIA2300u (8.7 kb)	pCAMBIA2300 (www.cambia.org)	Plants	None	Stable transformation. Suitable for molecular complementation studies. Kanamycin resistance.
4	pCAMBIA3300u (8.4 kb)	pCAMBIA3300 (www.cambia.org)	Plants	None	Stable transformation. Suitable for molecular complementation studies. Basta resistance.
5	pPS48u (3.5 kb)	pPS48 (12)	Plants	35S	Transient expression. Ampicilin resistance.
6	pCAMBIA130035Su (9 kb)	pCAMBIA1300 (www.cambia.org) and pPS48 (12)	Plants	35S	Stable transformation. Suitable for overexpression studies. Hygromycin resistance.
7	pCAMBIA230035Su (9.8 kb)	pCAMBIA2300 (www.cambia.org) and pPS48 (12)	Plants	35S	Stable transformation. Suitable for overexpression studies. Kanamycin resistance.
8	pCAMBIA330035Su (9.5 kb)	pCAMBIA3300 (www.cambia.org) and pPS48 (12)	Plants	35S	Stable transformation. Suitable for overexpression studies. Basta resistance.
9	pBGF-0u (15 kb)	pBGF-0 (13)	Plants	None	Stable transformation. For promoter-reporter analysis with a nuclear localized GFP variant. Kanamycin resistance.
10	pNB1MRGSHis6u (3 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For N-terminal fusions to a RGSHis6 tag. *Xenopus* β-globin UTRs. Ampicilin resistance.
11	pNB1YFPu (3.8 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For N-terminal fusions to YFP(17). *Xenopus* β-globin UTRs. Ampicilin resistance.
12	pNB1YNu (3.5 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For N-terminal fusions to the N-terminal part of YFP for use in BiFC (17,18). *Xenopus* β-globin UTRs. Ampicilin resistance.
13	pNB1YCu (3.3 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For N-terminal fusions to the C-terminal part of YFP for use in BiFC (17,18). *Xenopus* β-globin UTRs. Ampicilin resistance.
14	pNB1uYFP (3.8 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For C-terminal fusions to YFP (17). *Xenopus* β-globin UTRs. Ampicilin resistance
15	pNB1uYN (3.5 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For C-terminal fusions to the N-terminal part of YFP for use in BiFC (17,18). *Xenopus* β-globin UTRs. Ampicilin resistance.
16	pNB1uYC (3.3 kb)	pNB1u (this work)	*X.laevis* oocytes	T7	For C-terminal fusions to the C-terminal part of YFP for use in BiFC (17,18). *Xenopus* β-globin UTRs. Ampicilin resistance.
17	pPS48YFPu (4.3 kb)	pPS48u (this work)	Plants	35S	Transient expression. For N-terminal fusions to YFP (17). Ampicilin resistance.
18	pPS48YNu (4 kb)	pPS48u (this work)	Plants	35S	Transient expression. For N-terminal fusions to N-terminal part of YFP for use in BiFC (17,18). Ampicilin resistance.
19	pPS48YCu (3.8 kb)	pPS48u (this work)	Plants	35S	Transient expression. For N-terminal fusions to the C-terminal part of YFP for use in BiFC (17,18). Ampicilin resistance.
20	pPS48uYFP (4.3 kb)	pPS48u (this work)	Plants	35S	Transient expression. For C-terminal fusions to YFP (17). Ampicilin resistance.
21	pPS48uYN (4 kb)	pPS48u (this work)	Plants	35S	Transient expression. For C-terminal fusions to the N-terminal part of YFP for use in BiFC (17,18). Ampicilin resistance.
22	pPS48uYC (3.8 kb)	pPS48u (this work)	Plants	35S	Transient expression. For C-terminal fusions to the C-terminal part of YFP for use in BiFC (17,18). Ampicilin resistance.

A range of USER vectors has been generated by insertion of PacI. Cassettes (denoted by a 'u') into multiple cloning sites of established vectors (vectors 1–9). The vectors have been used further to generate a comprehensive set of translational fusion vectors (vectors 10–22) by sequential USER cloning. Abbreviations: USER cloning, see Materials and Methods section for definition. BiFC, bimolecular fluorescence complementation, UTR, untranslated region, YFP/GFP, yellow/green fluorescence protein.

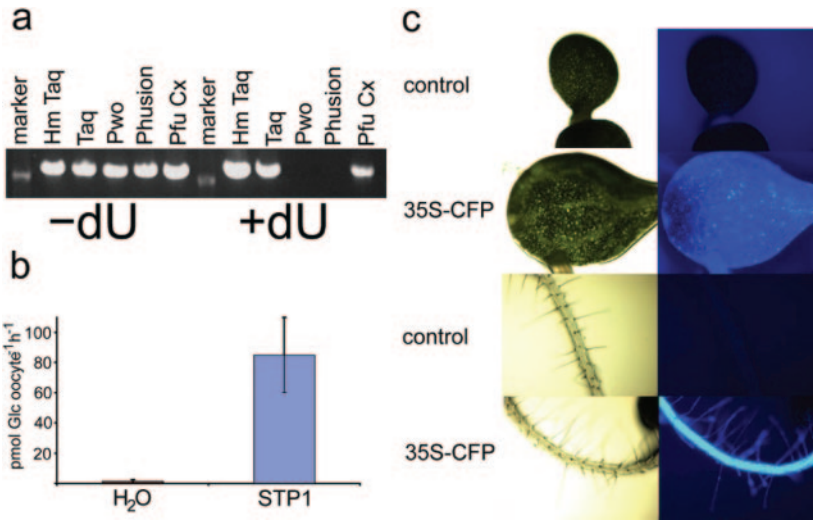

Figure 2. Characteristics and applications of the USER technique. (**a**) A comparison of the ability of DNA polymerases to amplify DNA fragments using uracil-free (-dU) and uracil-containing primers (+dU). Hm Taq: HotMaster™ *Taq* DNA Polymerase, Taq: Platinum® *Taq* DNA Polymerase, Pwo: Pwo DNA Polymerase, Phusion: Phusion™ DNA Polymerase, PfuCx: PfuTurbo® C_x Hotstart DNA polymerase. (**b**) Functional expression of *A.thaliana* glucose transporter AtSTP1 in *X.laevis* oocytes from the *Xenopus*-specific USER compatible vector, pNB1u. H_2O: control oocytes injected with water; STP1: oocytes injected with *AtSTP1* cRNA. (**c**) Functional expression of the cyano fluorescence protein in leaf and root of *A.thaliana* from the CaMV 35S promoter using the USER compatible pCAMBIA230035Su vector (35S:CFP). Control: empty pCAMBIA230035Su.

lower the stringency by which DNA fragments are inserted in a directional fashion. However, we have cloned more than 500 PCR fragments by USER cloning into the described PacI cassette and have not detected a single fragment that has been inserted in the wrong direction (data not shown). Furthermore, calculated T_m values for the hybridizing ends of fragments inserted in the correct orientation are ~22°C (11) while the corresponding T_m's for fragments hybridizing in the wrong orientation are close to 0°C. This indicates that the single base difference between the otherwise identical halves of our PacI cassette is sufficient to ensure directional cloning.

We have sequenced ~60 kb and identified only three errors, which confirmed the proof reading activity of the PfuCx polymerase.

Conversion of established vectors to USER vectors

By inserting the described PacI cassette into multiple cloning sites of established vectors, a collection of USER vectors was constructed (Table 1). This included vectors for constitutive expression in *Pichia pastoris* (AOX1 promoter and Zeocin resistance), *Xenopus* oocytes (T7 promoter, β-globin UTRs and ampicilin resistance), and *in planta*. The latter included vectors based on the Cauliflower Mosaic Virus (CaMV) 35S promoter and nos terminator (12) using three different selection markers (kanamycin, hygromycin and basta). In addition, the PacI cassette was inserted into a plant vector designed for promoter-reporter analysis using a nuclear localized GFP variant (13) and into plant vectors suitable for molecular complementation experiments (Table 1).

PacI cassette functionality

When cloning into the generated USER vectors, which originated from already established vectors, a short 13 bp sequence was introduced between the inserted DNA fragment and neighboring vector sequences (Figure 1, bottom). To ensure that this short 13 bp sequence did not impede the transcriptional or translational machinery in host organisms, we functionally expressed the *A.thaliana* glucose transporter AtSTP1 (14) in *X.laevis* oocytes from the generated *Xenopus*-specific USER expression vector, pNB1u (15) and the CFP *in planta* from the plant-specific USER expression vector, pCAMBIA230035Su (Table 1). AtSTP1-expressing oocytes exhibited the expected glucose transport activity compared to uninjected oocytes (Figure 2b) (16). Similarly, several independent transgenic plants expressing CFP exhibited a clear fluorescence compared to empty vector controls (Figure 2c). This indicated that the 13 bp did not impede the transcriptional or translational machinery in the two different host organisms.

Sequential USER cloning of multiple insertions

The commercial USER cloning approach allows insertion of only one DNA fragment into compatible vectors. We have developed a method for sequential USER cloning of multiple fragments into a vector once this has had the 38 bp PacI cassette inserted. The method is based on including 25 bp of the PacI cassette in the sequence of the inserted DNA fragment either through PCR or in chemically synthesized fragments (Figure 3). Together with the 8 nt overhang and the remaining 5 bp of the Nt.BbvCI nicking site on the vector, these 25 bp will regenerate the PacI cassette upon insertion, and enable further USER cloning of fragments into this vector. Sequential USER cloning of DNA fragments into vectors is particularly useful for vector construction. As an example, a vector suitable for translational fusions to YFP was constructed by USER cloning the YFP gene into the USER compatible *Xenopus* expression vector, pNB1u (Table 1). By including 25 bp of the PacI cassette sequence in the reverse primer used to amplify the YFP gene, the full PacI

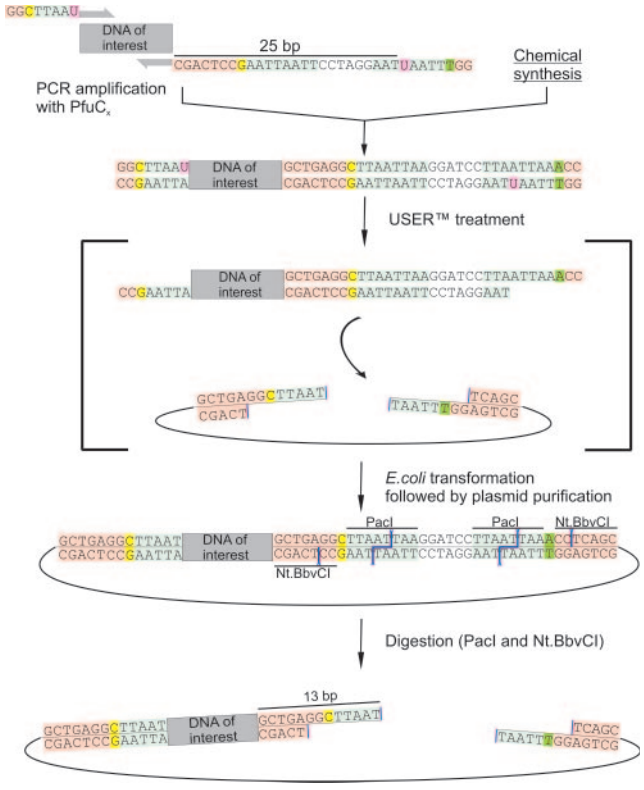

Figure 3. Sequential USER cloning of multiple inserts. Inclusion of 25 bp of the PacI cassette sequence in the reverse primer used to amplify a DNA fragment prior to USER cloning results in regeneration of the PacI cassette downstream of the inserted fragment. For smaller fragments the entire insert can be assembled from chemically synthesized oligonucleotides. Subsequent digestion of the construct with PacI and Nt.BbvCI allows insertion of another fragment into the vector by USER cloning. Sequentially inserted DNA fragments will have a minimum of 13 bp sequence between them. Nt.BbvCI recognition sites are marked in tan, PacI recognition sites are marked in light blue. Yellow and green mark the single base differences between the generated 3′ overhangs.

cassette was regenerated downstream from the YFP gene upon insertion of the fragment (exemplified in Figure 3, DNA of interest denotes the YFP gene). The distance between the YFP coding sequence and a subsequently inserted gene (through USER cloning) would be 13 bp (Figure 3) and would thus bring the inserted fragment out of the YFP reading frame. Therefore, the reverse primer used to amplify the YFP gene had an extra two cytidine residues added between the 25 bp sequence and the YFP ORF specific sequence (see Materials and Methods). This design ensured that subsequent digestion of the YFP containing pNB1u vector with PacI and Nt.BbvCI enabled USER cloning of DNA fragments into the vector downstream of and in frame with the YFP tag and hence would result in N-terminal YFP fusion constructs. With this approach, we inserted YFP (17), YFP-based bimolecular fluorescence complementation (BiFC) tags (18) and RGSHis6 tags into our existing USER vectors to construct a comprehensive set of C- and N-terminal translational fusion vectors for subcellular localization, protein–protein interaction and protein purification studies, respectively. For a full-list of our vectors, see Table 1. The efficacy of the vectors has been verified experimentally and had a cloning efficiency of approximately 100–200 colonies per 0.01 pmol of vector when mixed with at least 4-fold molar excess of PCR fragment. Moreover, the percentage of recombinant colonies exceeds 95% when the molar ratio of vector:PCR fragment is above 1:2 (data not shown). This is fully comparable to the cloning efficiency observed by NEB with their USER vector (http://www.neb.com/nebecomm/ManualFiles/manualE5500.pdf).

DISCUSSION

The uracil-excision based cloning technique has although it holds some very favorable features, not been widely applied as evidenced by the lack of citations in the literature. In this study, we have provided significant improvements which in our view have contributed to creating an ideal cloning technique for insertion of PCR amplified fragments into vectors. We have shown that the modified *Pfu* polymerase, PfuCx, is a highly suitable, proof-reading DNA polymerase to be used in conjuction with USER cloning. This polymerase was developed in 2002 in a study, which examined the structural basis for uracil inhibition of proof-reading polymerases via a targeted point mutation approach of the *Pfu* polymerase (10). Normally, uracil residues are introduced in DNA templates due to spontaneous deamination of dCTP to dUTP (19). Proof-reading enzymes are able to detect and stall at uracil residues in the template as these represent a promutagenic event generating G-C to A-T mutations (20). This stalling has deleterious effects on PCR product yield where spontaneous deamination of dCTP occurs more frequently during temperature cycling (19). In this context, PfuCx has been promoted as a proof-reading polymerase which, due to its ability to read through uracils, has a more robust performance and a higher yield. In this study, PfuCx is shown to be able to utilize uracil containing primers to amplify DNA fragments with high-fidelity for cloning purposes with the uracil excision technique. By identifying a USER compatible proof-reading DNA polymerase, we have overcome a major drawback of the currently existing USER™ cloning technique. It is important to note that DNA fragments amplified with the PfuCx polymerase for USER cloning will be susceptible to have deaminated dCTPs incorporated in the template. Due to the high fidelity of the PfuCx polymerase only few errors have been observed in our sequencing of USER cloned DNA fragments and thus do not provide sufficient data to show whether G-C to A-T substitutions occur more frequently when using PfuCx. Nevertheless, it is possible that the fidelity of the system could be enhanced by including a dUTPase in the polymerase enzyme mix. Thereby, generated dUTPs would be degraded to dUMPs which cannot be incorporated in DNA strands during PCR (19).

A second improvement to the existing commercial USER™ technique, is the advancement of the vector design strategy. First, the cassette used to convert vectors was modified to accommodate a much wider range of vectors. The vector conversion approach developed by NEB involves insertion of XbaI-containing cassettes into the multiple cloning sites of vectors. This imposes a limit on the range of convertable vectors as statistically XbaI recognition sites occur every 4096 bp and therefore can be expected to be present in many vectors. This is particularly a problem when working with large vectors, such as those required for plant

transformation. By developing a novel cassette containing the 8 bp PacI recognition site, which statistically only occurs every 65 536 bp, this problem has significantly been reduced, while at the same time preserving the directional insertion of the DNA fragments. Second, we have developed a strategy for sequential USER cloning of multiple inserts into the same vector. The method relies on regenerating the PacI cassette when USER cloning fragments into the first cassette. This is done by including a large part of the PacI cassette sequence in the inserted DNA fragment. Thus, for any given vector design only one initial restriction-ligation based insertion of a PacI cassette is necessary. This feature greatly facilitates the construction and modification of any vector, and is particular useful when working with large vectors which commonly have a very limited selection of unique restriction sites. Furthermore, it enables researchers to keep up with the rapid development in e.g. peptide tags by allowing easy upgrading of their favorite vectors.

The improved USER cloning technique combines several advantageous features from various existing cloning techniques. DNA fragments are directionally inserted into destination vectors by an approach, which is as straightforward and fast as that of the commercial USER™ cloning technique while having the high fidelity enjoyed by most other established cloning techniques. The simplicity of the USER cloning technique is comparable to that of topoisomerase-assisted cloning (21), while the low cost per cloning reaction is comparable to that of classical restriction-ligation based cloning. With respect to the improved design strategy of USER vectors the transparency, flexibility and inexpensiveness endows the researcher with complete control over the vector design independent of commercial vendors. This is demonstrated by our ability to rapidly generate a comprehensive set of vectors covering a wide range of applications, such as constitutive expression in a variety of host organisms as well as protein–protein interaction and subcellular localization studies (Table 1). Furthermore, because the same PacI cassette has been used to generate all our USER vectors, any fragment that has been PCR amplified with primers containing the described 8 bp tails can be inserted into all our vectors, much like in the concept of recombination-based Gateway® system (Invitrogen), where fragments which have been inserted into entry clones can be transferred to many destination vectors. In comparison to the currently dominating Gateway® cloning technique, the improved USER cloning technique presents several advantages when cloning a PCR fragment into a vector. Primers for PCR need to have tails of only 8 bp compared to 29 bp for Gateway® primers (http://www.invitrogen.com/content/sfs/manuals/ gateway_clonaseii_man.pdf), which represents a significant reduction in risk of errors and in cost. In addition, in the USER technique fragments are inserted directly into destination vectors while Gateway® requires an extra, time-consuming subcloning step via the entry vector. However, the entry clone concept of Gateway® is advantageous to the USER technique when needeeding to transfer the exact same PCR fragment into many vectors. Via recombination, fragments are transferred into the different destination vectors without the need for further sequencing while each cloning event in the USER technique involves insertion of a PCR product, and consequently requires sequence verification. However, the simplicity, high efficiency, fidelity, low cost and flexibility of the improved USER technique compensate for this by overall reduction in laboratory work time. There is an increasing demand for high-throughput methods for insertion of PCR fragments into vectors, e.g. for creating clone libraries. The improved USER cloning technique is exceptionally powerful in this respect, which was evident in a recent study where we transferred 216 different *A.thaliana* genes, into a USER-compatible vector with an efficiency as high as 96% (H. H. Nour-Eldin, M. H. H. Nørholm and B. A. Halkier, manuscript submitted). The entire procedure took approximately one week and included two rounds of validation via PCR on colonies where we tested one colony per plate per round of validation.

Moreover, the procedure proved to be very easy to automate. In this way, the Gateway® technique and other recombination based cloning techniques could benefit from the improved USER technique when generating similar libraries by inserting the PacI cassette between recombination sites in their entry vectors. Finally, when sequentially USER cloning multiple fragments into our USER vectors to generate e.g. translational fusion constructs only 15 bp will remain at the junction between the inserted fragments. Thus, even though the improved USER technique is not completely seamless, it represents a significant improvement compared to the Gateway® system, which leaves a minimum of 24 bp (22). Moreover, the approach described in this study for generating translational fusions is flexible and may be improved by using other combinations of restriction and nicking enzymes in the cassette.

In conclusion, the ability to use a proof-reading DNA polymerase to amplify DNA fragments for USER cloning experiments in combination with our vector design approach has, in our view, generated an ideal technique for cloning PCR fragments into vectors.

With completion of the genome sequencing projects, the next major challenge is to identify the functional role of the genes. High-throughput techniques to directionally transfer error-free DNA fragments into a given vector represent a critical bottleneck. The improved USER technique provides the means to efficiently overcome this bottleneck and based on the impact on the cloning throughput in our laboratories, we believe that this technique will be able to move molecular biology into an era where the cloning step occupies only a minor part of a molecular research project.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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